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Supplementary Information

RTG-loaded Nanomicelles (CS-VES-NAC) for Oral Anti-HIV Drug Delivery

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Experimental Section

Materials.

RTG was purchased from HuiKangBoYuan Chemical Tech Co., Ltd. (Beijing, China). Chitosan (Mw 150 kDa, degree of deacetylation 93.2%) was purchased from JinQiao Biochemical Co. Ltd. (Shandong, China). Vitamin E succinate (VES) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N-Acetyl-L-cysteine (NAC) was obtained from Tianjin Heowns Biochem LLC. (Tianjin, China). 1-Hydroxybenzotriazole (HOBT) and 1-Ethyl-3-(3-dimethylamiNopropyl) carbodiimide hydrochloride (EDCI) were purchased from Beijing Ouhe Technology Co., Ltd. All other chemicals were analytical grade and used without further purification.

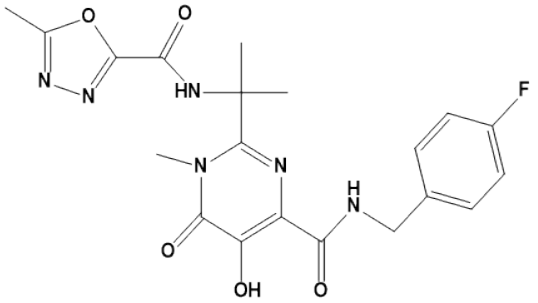

Figure S1 The chemical structure of raltegravir.

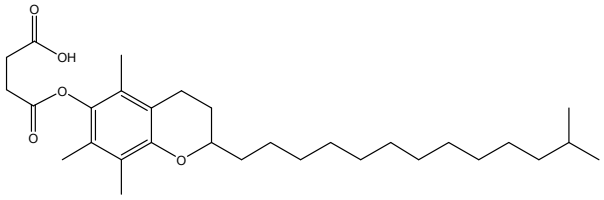

Figure S2 The chemical structure of VES.

Methods.

Synthesis of CS-VES. CS-VES copolymer was synthesized, according to the previous report,[3] by connecting the carboxyl-group of VES to the amino-group of chitosan in the presence of EDCI. A schematic representation of the reaction was shown in Figure S2A. VES (600 mg in 25 mL anhydrous ethyl alcohol) was added into a solution of CS (200 mg) in 30 mL deionized water, and then an appropriate amount of EDCI was added. After 5 hours, stirring at 70 ºC, the reaction was continued at room temperature for another 24 hours. The product was purified by dialysis against a dialysis membrane (molecular weight cutoff MWCO 10 KDa; Biosharp, Hefei, China) in 50% ethanol solution to remove hydrophobic remains, and then dialyzed in deionized water to get rid of hydrophilic byproducts. After that, the product was lyophilized and the soft white solid was obtained.

Synthesis of CVN. NAC was first activated by EDCI in anhydrous alcohol and CS-VES was catalyzed by HOBT in the deionized water. Subsequently, the activated NAC was dropwise added into the CS-VES solution and the pH was adjusted to 5.0 by adding NaOH. The reaction was carried out at room temperature for 3 h and protected from light (Figure S2A). The product was purified by dialyzing in 5 mmol/L HCl containing 1% NaCl solution at 4 ºC in dark. Finally, the polymer solution was lyophilized and kept at 4 ºC for

later use.

Nanoparticle preparation. Multifunctional CVN nanomicelles were prepared by a probe-type ultrasonic method using a nanoprecipitation/dispersion technique.[1] 6 mL of CVN copolymer solution was first prepared at a concentration of 1 mg/mL. RTG was dissolved in 0.5 mL dehydrated acetonitrile, and then the RTG solution was added to the CVN solution with stirring. Then, the dehydrated acetonitrile was removed by vacuum evaporation. The mixture was sonicated by a probe-type sonifier (VCX 750, Sonics, China) for 20 min in an ice bath. After that, the nanomicelles solution was centrifuged (3500 rpm, 10 min) and passed through a 0.8 μm filter to remove the unloaded drug and other impurities. The obtained CVN nanomicelles were then kept at 4 ºC.

Characterization. The molecular weight of CVN was measured by ubbelohde viscometer in 30 ± 0.5 ºC.

The chemical structure of CVN was analyzed by a Bruker ^{1}H NMR instrument (400 MHz, Germany) using tetramethylsilane (TMS) as an internal standard, and D_2O as solvents.

Fourier transform infrared spectroscopy (FTIR) was recorded on a Bruker Vertex 70 FTIR Spectrometer (Bruker Corporation, Germany) between 4000 and 600 cm^{-1}. The morphology of the nanomicelles was analyzed using a Tecnai G2 F20 U-TWIN transmission electron microscope (FEI, USA), working at 15 kV~200 kV. A drop of micellar solution was stained by 1% (w/v) uranyl acetate, and dropped in a copper grid, and then the liquid was blotted off and air-dried before measurement.

Sizes and zeta potentials of CVN nanomicelles were measured with a Zetasizer Nano ZS dynamic light-scattering (DLS) instrument (Malvern, U. K.) at 20 °C at an angle of 90°.

Elemental analyses were performed on a Elementar Vario EL CUBE (Germany) Elemental analyzer.

RTG-loaded. RTG was encapsulated in the co-assembly via a probe-type ultrasonic method utilizing a nanoprecipitation-dispersion technique. Obtained mixture of RTG and RTG-loaded CVN was centrifuged (3500 rpm, 10 min) to remove excess RTG. The encapsulation efficiency (EE) and drug loading capacity (DLC) of RTG-loaded nanomicelles were measured by high performance liquid chromatography (Agilent, Germany). In short, RTG-loaded nanomicelles solution was diluted 10-fold with methanol and sonicated to destroy the nanomicelles and dissolve RTG. The samples were then transferred to a sample vial after filtration with 0.22 μm filtration membrane. Chromatographic separation was handled in the reverse-phase ODS Diamonsil-C_{18} column (250 mm × 4.6 mm, 5 μm) at 20 ºC. The mobile phase consisted of deionized water and HPLC grade methanol (25:75, v/v). The flow rate of the sample in the chromatographic column was 1 mL/min and the wavelength of ultraviolet-visible detector was 320 nm. The EE% and DLC% of RTG were calculated by Eq. (1) and Eq. (2), respectively.

$EE\% = (RTG_t - RTG_f)/ RTG_t \times 100$ Eq. (1)

$DLC\% = (RTG_t - RTG_f)/ L_t \times 100$ Eq. (2)

Where RTG_t is the total amount of RTG, RTG_f is the free amount of RTG in dialysate, and L_t is the total weight of co-assembly.

Stability and stimuli-responsiveness. To study the stability of CVN nanomicelles, the samples were incubated at 37 ºC in a shaker, and the size was determined every half an hour. In addition, the nanomicelles were diluted in deionized water and balanced for 1 h before particle size measurement. In order to investigate the stimuli-responsive properties of nanomicelles, HCl, NaOH or fetal bovine serum (FBS) were added into the nanomicelles solutions to simulate gastric juice (pH~1.2), the extracellular acidic (pH~6.8) or blood environments (pH~7.4, containing serum protein), pH 7.0 was used as blank control. All samples were incubated at 37 ºC with shaking (110 r/min), and the size was monitored.[2]

In vitro release. In vitro release studies were performed using the method of dialysis. The RTG-loaded nanomicelles were dispersed in deionized water containing 2% Tween-20, which can improve the solubility

of RTG in variety of pH aqueous solution. Then, 2 mL dispersion was sealed in a dialysis bag and incubated in 30 mL of release medium at 37 ºC under orbital shaking (100 r/pm). At designated intervals, 2 mL samples were removed for analysis and replaced with the same volume of PBS. The RTG content was determined under the same HPLC conditions as described above.

Cytotoxicity assay. The cytotoxicity of CVN nanomicelles were evaluated using TZM-bl cells in comparison with a RTG formulation. TZM-bl cells were seeded into a 96-well plate (6.0×10^3 cells/well) and cultivated for 12 hours at 37 ºC. The cells were then coincubated with CVN, RTG or RTG-loaded CVN at 0.001, 0.01, 0.1, 1, 10, and 100 μg/mL equivalent RTG concentration and the same amount of solvent acetonitrile as control for 24, 48, and 72 hours. At selected intervals, 100 μL Cell Counting Kit-8 (CCK-8) (2 mg/mL) was added to each well and incubated for a further 1 hour. The absorbance at 450 nm was measured using a microplate reader (TECAN M200, Switzerland). Each sample was analysed in triplicate. The cell viability was defined by the following equation:

$$Cell\ viability(\%) = \frac{OD_s}{OD_{control}} \times 100\%$$

Where OD_s was the optical density of the cells incubated with CS-VES nanomicelles, and $OD_{control}$ was the optical density of the cells treated only with culture medium.

Cell Viability Tests. Cell viabilities were evaluated by measuring the mitochondria function by MAGI test. TZM-bl cells were dissociated by trypsin, seeded in 96-well plates at the concentration of 6.0×10^3 cells/well in 100 μL medium, and then incubated for 48 h before fixed. After staining, 100 μL PBS was added to each well, and Olympus Research Inverted System Microscope was used to count the number of locus coeruleus. Half-maximal inhibitory concentration (IC_{50}) was carried out by curve fitting of the cell viability data.

References

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